

COULD TANOAK MORTALITY AFFECT INSECT BIODIVERSITY?  
EVIDENCE FOR INSECT POLLINATION IN TANOAKS

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ABSTRACT

Tanoaks, *Notholithocarpus densiflorus* (Hook. & Arn.) Manos, Cannon & S. H. Oh, are being killed by sudden oak death, caused by the pathogen *Phytophthora ramorum* Werres, de Cock & Man in't Veld. However, very little is known about the basic ecology of the species. Here we investigate the pollination ecology of tanoaks using insect-visitor observations along with a pollinator-exclusion study. Insect-visitor observations were conducted by citizen-scientist volunteers at three different sites in the Midpeninsula Regional Open Space District lands in the Coast Range of California in 2009. Pollinator exclusions were conducted over two years (2009, 2010), using veil bags to prevent insects from reaching female flowers at the Blodgett Forest Research Station in the Sierra Nevada foothills. Microsatellite markers were used to infer selfing or outcrossing for the developing acorns. The citizen scientists observed 148 insect visitors to tanoak flowers over 11.5 hours of observation (in 65 observation periods). Pollinator exclusion resulted in lower fruit set and higher rates of selfing. The data suggest that tanoak is primarily an insect-pollinated species, though some level of wind pollination is likely. There is a diverse community of insects visiting tanoak flowers. In order to understand the importance of tanoaks to the native insect community, future research needs to focus on identifying the composition of the insect community, and the extent to which they rely on tanoak pollen and nectar as a food source.

Key Words: Flowering phenology, insect observations, insect pollination, *Lithocarpus densiflorus*, *Notholithocarpus densiflorus*, pollinator exclusion, tanoak.

Tanoaks, *Notholithocarpus densiflorus* (Hook. & Arn.) Manos, Cannon & S. H. Oh (Fagaceae), across the California Coast Range are being killed at an alarming rate by sudden oak death (SOD) (Meentemeyer et al. 2008), caused by the pathogen *Phytophthora ramorum* Werres, de Cock & Man in't Veld (Werres et al. 2001; Rizzo et al. 2002), with concerns being raised for the future of the species (Cobb et al. 2012; Cobb et al. this volume). This is particularly troubling as tanoaks are an important member of the California coastal forest ecosystem. Their acorns are an important food source for mammals and birds (Immel 2006), and their roots support a diverse community of fungi (Bergemann and Garbelotto 2006). However, the importance of tanoaks in the ecosystem has not always been acknowledged, and often it has been labeled as a "trash tree." As a result, the majority of research on tanoaks has focused on how to kill it using herbicides (reviewed in Bowcutt 2011). Until recently, very little was known about tanoak phylogeography, breeding system, or population structure (Nettel et al. 2009; Dodd et al. 2013). Much is unknown about the basic biology of the species, including how pollen is moved from one

tree to another. Here we present data suggesting that tanoaks may also play an important role in supporting pollinator communities.

Pollen can be moved between trees in a number of ways including wind, insects, mammals, or birds (Proctor et al. 1996). Understanding how pollen is carried between flowers helps to shed light on a number of important features of the biology of a plant. How far does the pollen travel? What is the size of its genetic neighborhood? What is the expected genetic structure of the species? How should conservation reserves be designed?

Tanoaks have been generally assumed to be wind pollinated. They have always been closely associated with members of the true oaks (*Quercus* spp.) and were first attributed to the genus *Quercus* (reviewed in Bowcutt 2011). Tanoaks have flowers similar to most other oak species, which are indeed wind pollinated (Proctor et al. 1996), except that male inflorescences (aments) in tanoak are erect, similar to insect-pollinated chestnut aments. An ament contains many staminate (male) flowers, and can also contain pistillate (female) flowers at the base (Fig. 1). The idea that the flowers are wind pollinated has never before been tested.



FIG. 1. A flowering branch from a tanoak tree at Blodgett Forest Research Station.

Many insect species are dependent on pollen as an important source of protein in their diet—particularly many bee species—irrespective of whether or not pollen is transferred from male to female flowers (pollination). If some insects rely on the flowers for pollen or nectar, then the role of tanoaks in the ecosystem has been underestimated, and the insect community will be impacted in ways not yet understood by the loss of tanoak trees due to SOD.

Here we present data from two complementary lines of investigation to address the following questions:

1. Do insects visit tanoak flowers in the field?
2. When insects are excluded from visiting flowers, do acorns still develop? How does insect exclusion impact selfing rates?

We took this two-pronged approach because observational studies are excellent at determining the community of insects that is visiting the flowers. However, they do not provide information on whether or not those visits result in pollen being transferred from one tree to another—i.e., whether or not pollination took place. In order to

determine that, it is necessary to exclude insects from flowers and determine if the rate of fruit set is similar with and without insect visitors.

## METHODS

### Insect Visitors

During July and August of 2009, a team of nine volunteer citizen scientists spent 11.5 hrs observing insect visitors to tanoak flowers at three sites in the Midpeninsula Regional Open Space District (MROSD): Windy Hill, Long Ridge, and El Corte de Madera Creek in San Mateo Co., California, an area heavily impacted by SOD. The volunteers were coordinated by MROSD staff and were given detailed written instructions on how to conduct the insect observations. Each volunteer recorded the following information: date; time; approximate temperature; and whether or not the focal flowers were in the sun, shade, or dappled light. They then selected a set of focal aments (from 1–36) consisting mainly of male flowers, which represented a group of flowers that they could comfortably watch all of during the observation

period. Each observation period lasted typically for 10 minutes. Each visit by an insect during the observation period was then recorded. A visit was defined as an insect coming into contact with the flower, and then departing. Sixty-five different observation periods were recorded. Volunteers were asked to categorize visitors as bees, flies, butterflies, beetles, ants, or birds. If they could not distinguish a visitor as a bee or a fly, they were asked to record it in a separate category: "bee/fly?"

*Insect observations data analysis.* To simplify the analyses and to account for non-random variation between observers, each observation period was assigned a "yes" or "no" value according to whether or not an insect visitor was observed (both for total visitors and by individual insect type). To summarize the data, stacked bar charts showing the frequency of each type of insect visitor were made. The charts do not contain error bars as they reflect observation counts made by the observers.

#### Flowering Phenology at the Blodgett Forest Research Station, Eldorado Co. (2009)

At a second field site in the foothills of the Sierra Nevada (an area uninfested by SOD), we observed bud, shoot, and flower development weekly throughout the flowering season, beginning in May 2009 and continuing through July 2009.

#### Floral-bagging Experiment and Insect-exclusion Experiment (2009)

At the Research Station, Eldorado Co., CA, we bagged five clusters of inflorescences on each of two branches per tree for 10 trees on June 1, 2009 (Fig. 2). Numbers of aments per cluster were variable. Bags were made from 1-mm mesh, wedding-veil material sewn closed and wrapped tightly around the branch to prevent insect entry. The mesh size was large enough to allow wind-blown pollen to enter (Neal and Anderson 2004). Although the mesh may have impeded some wind-blown pollen from entering, we expected ample pollen to be available because of the large number of tanoaks in the plot. The bags were tightly attached to the branches, but were loose around the inflorescences, so that damage to flowers or developing fruits is likely to have been minimal. At the same time we marked a similar number of inflorescences that were un-bagged, to serve as controls. On June 15, 2009, some of the male flowers were shedding pollen.

Bags were removed at the end of the flowering season in September 2009 and acorn development was observed in October 2010 after the two-year maturing period of tanoak acorns. Because the



Inflorescence in wedding veil

FIG. 2. An example of the pollinator exclusion bags excluding pollinators from visiting tanoak flowers.

numbers of pistillate aments varied among clusters of aments and the numbers of involucre per pistillate ament varied, we adjusted the observed numbers of acorns set for the observed numbers of pistillate aments per cluster and assumed an average of two involucre per pistillate ament. Differences in proportions of acorns set were tested with one-way analysis of variance, with bagging as the main factor and trees as replicates with branch data averaged per tree.

To test that these acorns were successfully pollinated, they were dissected to determine the presence of an embryonic plumule and radicle. A small piece of the cotyledon of each acorn was excised for DNA extraction. DNA of the cotyledons and foliage of mother trees was extracted and amplified using a set of nine microsatellite primers in order to determine whether the progeny were the result of selfing or outcrossing (for extraction and PCR protocols see Nettel et al. 2009).

#### Insect-exclusion and Emasculation Experiment (2010)

The bagging experiment that we performed in 2009 was intended to test whether tanoak flowers were predominantly insect pollinated. We genotyped acorns to assess selfing, but mutations and allele scoring errors could confound the results. Therefore, in this second study we repeated inflorescence bagging, but nested emasculation treatments within the bagging study. We bagged six clusters of inflorescences per tree for 20 trees on May 21, 2010. Male aments were removed

from three clusters per tree at the time of bagging, or as the aments extended. For combined pistillate–staminate aments, the distal staminate portion was removed. The remaining three clusters per tree were not emasculated to serve as controls of the effects of emasculation. In addition, we marked six clusters of inflorescences per tree that were un-bagged, three of which were emasculated and three of which were left intact. We removed the bags in October 2010, and acorn development was observed in October 2011. Differences in proportions of acorns set were tested with a two-way nested analysis of variance, with bagging as the main factor and emasculation nested within bagging and trees as replicates. Data were arcsine square-root transformed, by first taking the square root and then taking the arcsine of the square-root transformation.

A portion of the acorn cotyledon was excised for DNA extraction and amplification as for the 2009 experiment.

#### Estimating Selfing/Outcrossing

Because we had maternal genotypes for each of the acorns that were genotyped, and we had not genotyped potential paternal trees in the vicinity of the sample trees, we manually tested for evidence of outcrossing. Acorns were considered to be of outcross origin if at least one of the alleles at a locus was different than either of the alleles at that locus in the mother tree. We recognize that this does not account for potential mutations and allele scoring errors.

## RESULTS

### Insect Visitors

In 65 observation periods, spanning 11.5 hours, the citizen scientists observed 148 visits to tanoak flowers by insects. Long Ridge had the largest number of observation periods with at least one visitor recorded (20 periods, or 58.8% of observations) (Fig. 3a). However, Windy Hill had the highest percent of observation periods with visits (10 out of 15, or 66.7%). At Windy Hill, only bees and flies were observed, while all insect-visitor types were observed at Long Ridge (Fig. 3b).

Flowers located in the sun showed a trend towards a higher proportion of observation periods that recorded a visit compared to flowers in dappled light or shade (Fig. 4a). The temperature at the site (cool, warm, or hot) did not influence the proportion of observation periods with an insect visitor (Fig. 4b), nor did the time of day (morning vs. afternoon) (Fig. 4c). However, beetles were observed only in the afternoon (Fig. 5a). The part of the flowering season did not influence visitation (Fig. 4d), with the

exception of beetles, which were not observed towards the end of pollen shed (Fig. 5b).

### Flowering Phenology at the Research Station

Inflorescences first appeared as the bud scales opened during the first week of May. At this stage, inflorescences were short and compact and took about 10 days to reach full size. The first inflorescences to expand were mostly exclusively pistillate. As the shoot continued to expand later in the season, combined pistillate and staminate inflorescences were formed. These combined inflorescences included: 1) a distal staminate ament that was a little shorter than the earlier staminate aments; and 2) 1–3 female flowers at the base. These later-season aments became fully expanded during the period in which the earlier staminate aments were still shedding pollen.

This double pattern of male flowering provides a broad period for pollen dispersal. We observed differences of as much as 18 days between the flowering phases in different trees in the population. This variation in phenology among trees means that female flowers for an early-flowering individual are likely to be pollinated from the early staminate flowers, whereas late-flowering individuals could still be pollinated from the combined pistillate–staminate aments. In general, the two phases of staminate ament production provide an extended period of pollen shedding, so that most female flowers have a strong likelihood of being pollinated.

### Insect-exclusion Experiment (2009)

Because the numbers of pistillate aments varied among clusters of aments and the numbers of involucre per pistillate ament varied, we adjusted the observed numbers of acorns set for the observed numbers of pistillate aments per cluster and assumed an average of two involucre per pistillate ament. Differences in the proportion of acorns set were highly significant ( $P < 0.001$ ). About 14% of involucre in bagged inflorescences produced fully swollen acorns by October 2011, compared to 65% of unbagged inflorescences (Table 1). These data indicate that 1) the proportion of successful pollinations per inflorescence was lower when insects were excluded; and 2) some flowers from which insects were excluded were fertilized, presumably either from self-pollen or wind-blown pollen penetrating the veil.

### Insect-exclusion Experiment (2010)

As in the first experiment, bagging to exclude insects resulted in a lower rate of acorn set ( $P < 0.001$ ), but did not prevent pollination entirely (Table 2). Emasculation resulted in reduced acorn set in both bagged and unbagged tests ( $P$

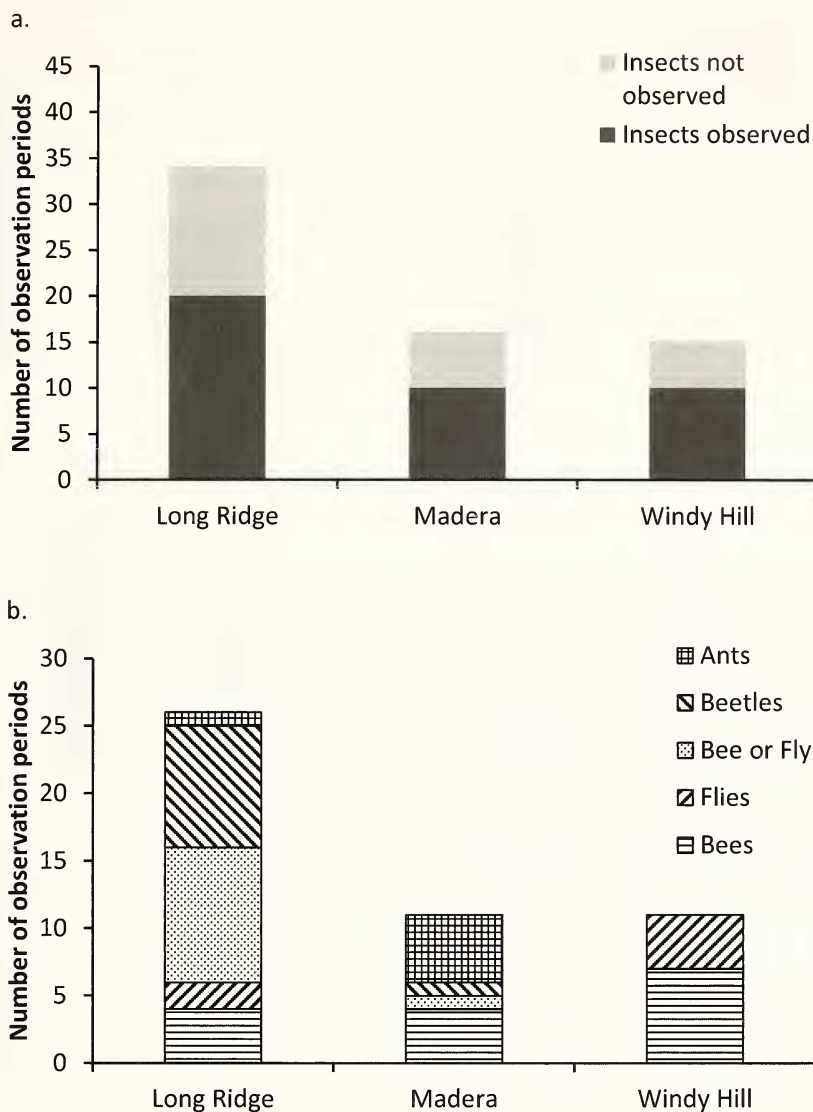


FIG. 3. 3a, total number of observation periods made at each site partitioned by whether or not insects were observed during each observation period; 3b, the number of observation periods at each site in which each of the four types of insect visitors were observed.

< 0.001). However, the relative effect of emasculation was greater in the bagged experiment than in the unbagged, suggesting pollen limitation because of the veil.

As expected, we did not detect any selfing among the bagged, emasculated inflorescences. However, a low proportion of selfed seeds were detected in the bagged, intact and in the unbagged, emasculated treatments (Table 2). The latter could be the result of geitogamous matings (pollen from other flowers on the tree). In total, three acorns were classed as selfed in the bagged, intact treatment and a single acorn was classed as selfed in the un-bagged, emasculated treatment.

## DISCUSSION

Tanoaks have been assumed to be wind-pollinated. Our data suggest that 1) tanoak is primarily an insect-pollinated species; and 2) some level of wind pollination is also possible.

These results could have important consequences for ecological conservation that need further investigation, both from the perspective of the insects and the trees. There could be a community of insects that are dependent on tanoaks as a food source, and the future reproduction of tanoaks could be somewhat dependent on those insects.

Pertaining to insect biology, it is first necessary to consider what the data do and do not show.

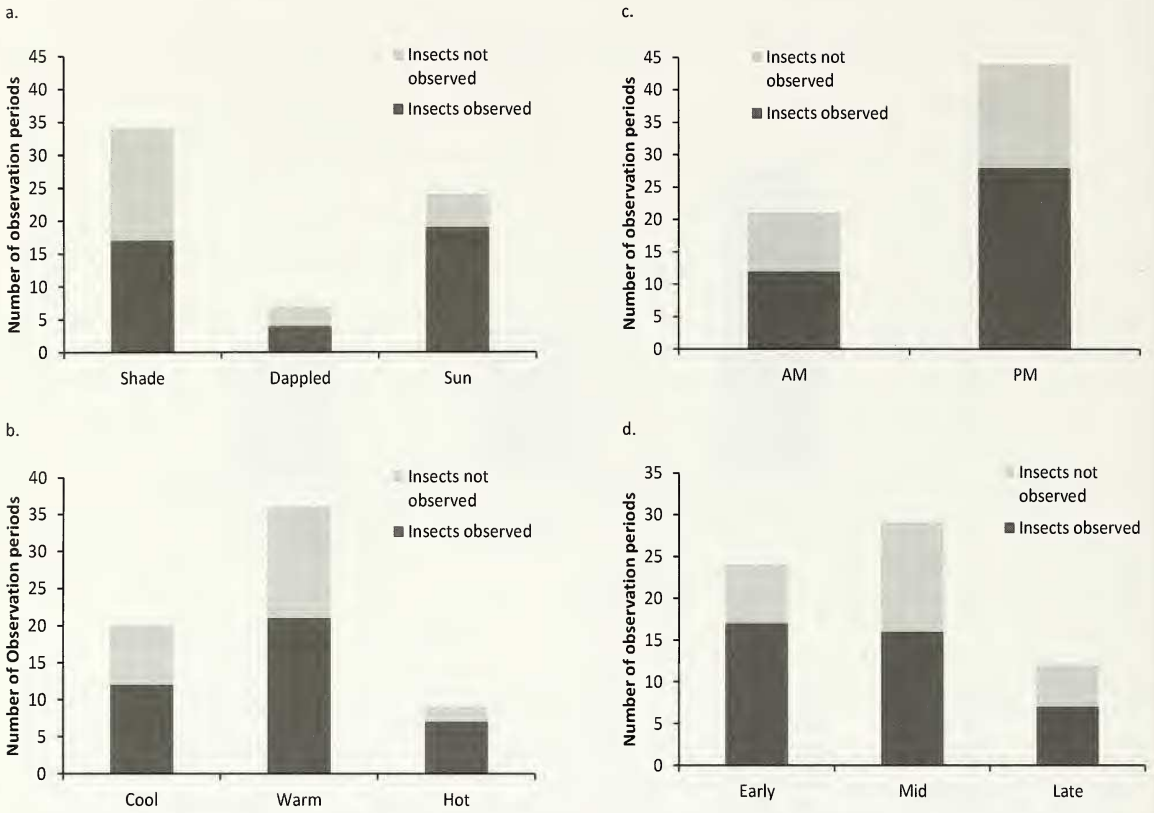


FIG. 4. Total number of observation periods in different environments; 4a, with flowers in each of the three different qualities of light: full shade, dappled light, or full sun; 4b, at three different temperature conditions: Cool  $\leq 75^{\circ}\text{F}$ , Warm =  $75\text{--}85^{\circ}\text{F}$ , Hot  $\geq 85^{\circ}\text{F}$ ; 4c, in the morning or afternoon; and 4d, over the course of the flowering season: Early = July 1–July 15, 2009, Mid = July 16–July 31, 2009, Late = August 1–August 15, 2009.

While the data do show that insects visit tanoak flowers, they do not show what the importance of tanoak pollen is in their diet. Moreover, the community of insects has not yet been characterized. How many different species use tanoak flowers? How many of those species are native? (Several non-native honeybees were observed in this study.) These questions are relevant for determining the importance of tanoaks for the native pollinator community, and thus the impacts that SOD would have if some areas become extirpated of tanoaks.

For trees, these results have some very clear implications for conservation, particularly in light of SOD. The movement of pollen across the landscape is an area of very active research (Austerlitz et al. 2004; Robledo-Arnuncio et al. 2006) that recently has overturned some of the classical paradigms of mainly local matings (Ashley 2010) and expected lower genetic diversity in fragmented populations (Kramer et al. 2008). Many studies have shown that the dispersal kernel is fat-tailed and some pollen can travel great distances—whether it be wind-pollinated as in Scots pine (*Pinus sylvestris* L.), in which pollen has been recorded to have traveled

and caused effective pollination over distances exceeding 100 km (Robledo-Arnuncio 2011), or insect-dispersed pollen over 160 km as in *Ficus sycamoros* L. (Ahmed et al. 2009). Indeed, fragmentation in many cases has only minor or non-significant effects on genetic diversity because of long-distance pollen dispersal (Kramer et al. 2008). However, the degree of fragmentation and the distance between fragments are likely to play a crucial role in individual cases and will be important in conservation of tanoak following disease epidemics. Although the dispersal kernel of pollen is important in the underlying ability to effect long-distance fertilizations, local matings are likely to result in genetic neighborhoods that will have important consequences on the selection of seed trees for replanting (Dodd et al. 2013).

In fragmented habitats, the size of the genetic neighborhood—and the distance traveled by pollen—can become increasingly important, as pollen must move between fragments in order to prevent a loss of genetic diversity in the remnant fragments (Ellstrand 1992; Kramer et al. 2008). In some areas tanoak populations are being decimated by SOD, and as such, are rapidly

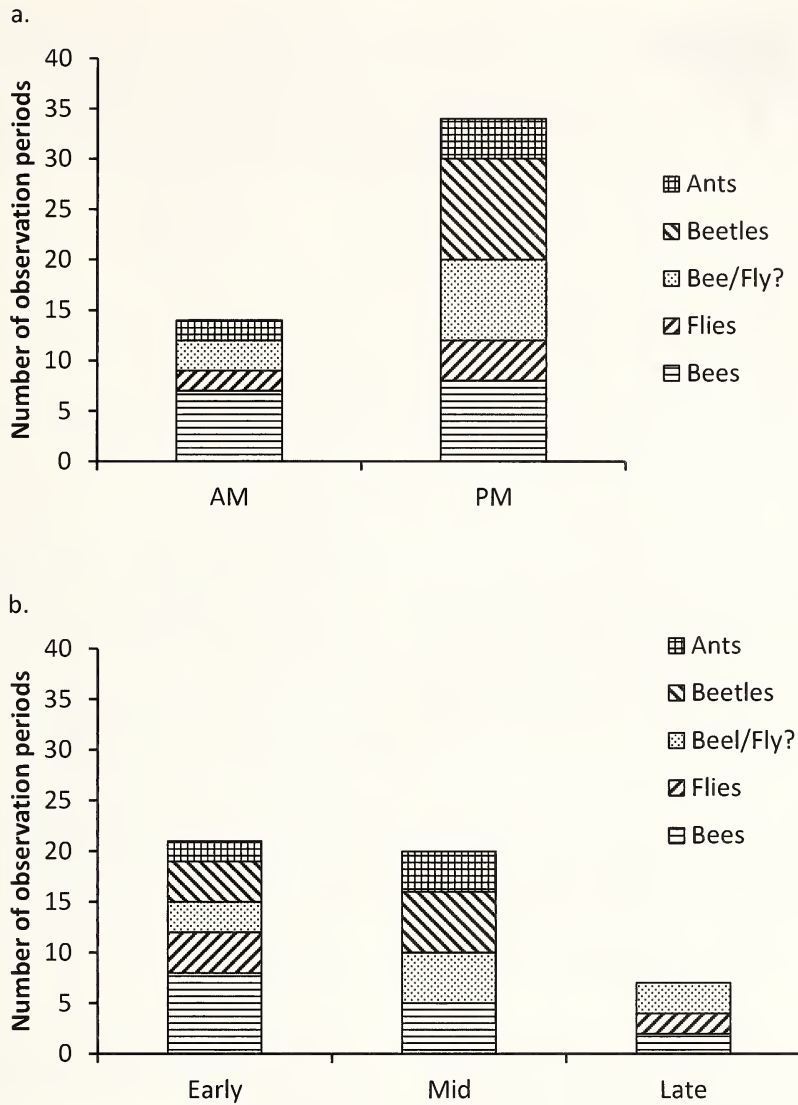


FIG. 5. The number of observation periods in which each of the four types of insect visitors were observed; 5a, in the morning or afternoon; 5b, over the course of the flowering season: Early = July 1–July 15, 2009, Mid = July 16–July 31, 2009, Late = August 1–August 15, 2009.

changing their patterns of fragmentation as populations split or steeply decline. Knowing that tanoaks rely on insect pollinators is critical to understanding the consequences of this recent fragmentation; should the distance between fragments exceed the flight distances of pollinating

insects, loss of genetic diversity could be an important consequence.

Further information that would be useful for informing conservation planning for surviving tanoak populations includes knowing the distance that pollen moves between individual trees

TABLE I. ACORN SET IN BAGGED INFLORESCENCES TO EXCLUDE INSECTS AND OPEN-POLLINATED (UNBAGGED) INFLORESCENCES IN 2009 EXPERIMENT. (Standard errors in parentheses.)

Treatment	Mean number of pistillate aments per cluster	Mean number of acorns set per cluster	Mean proportion of acorns set per cluster assuming two involucre per pistillate ament	Mean proportion of acorns set that were selfed per cluster
Bagged	6.3 (0.2)	1.78 (0.2)	0.14	0.02 (0.01)
Unbagged	7.1 (0.8)	9.24 (1.2)	0.65	0.04 (0.02)

TABLE 2. ACORN SET IN BAGGED INFLORESCENCES EMASCULATED AND INTACT TO EXCLUDE INSECTS AND OPEN-POLLINATED (UNBAGGED) INFLORESCENCES IN 2010 EXPERIMENT. (Standard errors in parentheses.)

Treatment	Mean number of pistillate aments per cluster	Mean number of acorns set per cluster (October 2012)	Mean proportion of acorns set per cluster assuming two involucre per pistillate ament	Mean proportion of acorns set that were selfed per cluster
Bagged (emasculated)	5.1 (1.7)	0.7 (0.08)	0.05 (0.005)	0
Bagged (intact)	7.6 (1.9)	1.6 (0.19)	0.11 (0.01)	0.02 (0.01)
Unbagged (emasculated)	7.2 (2.1)	7.2 (0.34)	0.50 (0.02)	0
Unbagged (intact)	6.5 (2.0)	11.7 (0.34)	0.77 (0.02)	0.002 (0.002)

and populations. What are the flight distances of the insects that pollinate them? Moreover, information on the composition of the pollinator community is critical, as conservation planning also needs to account for maintaining the populations of insects that visit tanoak flowers in order to assure maximum seed set.

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